Food Sci. Biotechnol. Vol. 14, No. 4, pp. 503 ~ 508 (2005)

Food Science and Biotechnology

Isolation and Characterization of Antilisterial Lactic Acid Bacteria from *Kimchi*

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Abstract Screening for antilisterial activity was performed in about three thousand isolates of lactic acid bacteria (LAB) from Chinese cabbage *kimchi*, and finally based on the relatively stronger antilisterial activities eight bacterial strains were selected. The bacteria were further characterized in terms of their tolerance to artificial gastric juice, pH 2.5, bile salts (0.3% oxgall), and to the different NaCl concentrations. Of the isolates, YK005 was especially investigated for its physiological characteristics due to its inhibitory activity against gram-positive *Listeria monocytogenes* as well as gram-negative *Escherichia coli* O157:H7, as they have been constantly reported to be resistant against bacteriocins produced by a number of LAB strains. YK005 was found to be rod-shaped, 3.8 μm long × 0.5 μm wide, non-sporeforming, non-motile, catalase-negative, and produced gas from glucose (heterolactic). Based on the biochemical data obtained by API 50 CHL medium, the isolate was tentatively identified as *Lactobacillus brevis*. To validate the result obtained by the biochemical identification, rRNA-based PCR experiments using a pair of species-specific primers for *L. brevis* were conducted and a single band of 1400 bp was observed, which strongly indicated that YK005 belongs to *L. brevis*. The LAB isolates are potentially exploited as human probiotic organisms and are employed to control some food-borne pathogens like *L. monocytogenes*.

Keywords: antilisterial activity, lactic acid bacteria, *kimchi*, probiotic, isolation, identification

Introduction

There are many types of *kimchi*, which is a traditionally fermented vegetable in Korea and there has been increasing consumption of factory-made products with current yearly rise in sale by 15-20%. According to the reported literatures (1-2), more than 200-kinds of *kimchi* are made home in a traditional way depending mostly on the raw materials used, processing methods, seasons, geographical locations, etc. When it comes to the raw materials, Chinese cabbage together in combination with radish is most frequently employed. Needless to say, the final product quality is significantly affected by the type of fermenting organisms involved in the process. For overall tastes, the best sensory results were obtained around pH 4.2 and after the development of 0.6% of lactic acid on the third consequent day after fermentation at 20°C (3). As the fermentation relies on the LAB originally present in the raw vegetable and various spices, it is generally believed that the process is occupied by heterolactic fermenters in the earlier stage and gradually replaced by the homolactic fermenters representing *L. plantarum* (4). Almost for two decades, many reports have been published on the microorganisms responsible for fermenting *kimchi* with emphasis on predominant flora during the series of processes including both anaerobic *L. plantarum, L. brevis, Streptoccus faecalis, Leuconostoc mesenteroides, Pediococcus cerevisiae*, and aerobic coliforms, *Achromobacter, Flavobacterium, Pseudomonas* (2). Also, at the later stage of fermentation, outgrowth of yeasts and moulds make significant contributions in deteriorating the overall quality of *kimchi* in terms of texture and flavors, which makes the products unacceptable for consumption. In general, heterolactic fermenters such as *Leu. mesenteroides* are predominant in the earlier stage, while homolactic fermenters representing *L. plantarum* are predominant in the later stage of fermentation (4-5). However, despite of extensive research focused on this issue over the past 50 years, there is not complete understanding on the distribution of members in the microbial community and limited information is available on bacterial successions especially with respect to the ecological aspects (6-7).

As *Listeria monocytogenes* is known to cause serious or even fatal illness and can survive for a long period of time at refrigerated temperature (8) and at sodium chloride concentrations of up to 10% (9), it is a serious health threat to humans, particularly by consumption of lightly preserved seafoods. Immense research efforts have been carried out for the discovery of new molecules, which could be the suitable targets for such types of foodborne pathogens. Since none of the studies have dealt with listeriosis directly originated from *kimchi* in Korea, some proper measures should be established to control this deadly pathogen from causing food contamination.

This study was carried out to screen the LAB isolates from *kimchi* for antilisterial activity, and to investigate whether or not the antilisterial isolates have relevant properties as a potential starter with probiotic function in causing fermentation of *kimchi*.

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Received March 16, 2005; accepted July 11, 2005

Materials and Methods

Strains, media, and isolation procedure As soon as the samples were received, they were properly diluted in 0.85%(w/v) saline and plated on an modified MRS agar (MMRS, 10) supplemented with 10%(v/v) cabbage juice and bromocresol pruple (BCP; Difco, Detroit, USA) as a pH indicator (11). Based on the colony morphology, distinct colonies were arbitrarily picked from the agar media. Each of the isolate was streaked twice on MRS agar (Difco) to obtain a pure culture. *E. coli* 0157: H7 (ATCC 35150) was propagated in McConkey broth (Difco). All the strains used in this experiment were purchased from the Korean Collection for Type Cultures (KCTC; Daejon, Korea) and Korean Culture Center for Microorganism (KCCM; Seoul, Korea), and transferred to fresh medium biweekly and kept in 20%(v/v) glycerol stock at -70°C until use. Also the eight antilisterial isolates were streaked twice on MRS agar (Difco) and were named as YK001, YK005, YK008, YK011, YK012, YK013, YK014, and YK015, respectively.

Assay of antilisterial activity The antibacterial activity of the *kimchi* isolates against a *L. monocytogenes* KCCM 40307 was evaluated on BHI agar (Difco) by using a paper disc diffusion assay (12). The LAB isolates were propagated in BHI broth twice at 37°C for 12 hr before experimental use. Bacterial growth was determined by measuring the optical density at 650 nm in triplicate and, when the stationary phase was reached (approx. 20 hr), 10 mL of cell-free supernatant of each LAB isolate was obtained by centrifugation (8000 × g, 5 min at 4°C), the pH was adjusted to 6.5 and was heat treated for 10 min at 80°C to inactivate protease activity. Bottom agar was prepared by adding 1.5%(w/v) agar to the BHI broth. The overlays used for bacteriocin assay were prepared with 0.7 % BHI agar. *L. monocytogenes* KCCM 40307 was propagated on MRS broth and kept in 20% glycerol stock at -70°C until use.

Morphological, physiological and biochemical tests For physiological characterization of the isolates, all the isolates were sub-cultured twice overnight in MMRS broth containing 10%(v/v) Chinese cabbage juice (10) at 30°C prior to the subsequent experiments. The gas production was tested using Durham tubes (Φ8 × 30 mm). Bacterial growth was monitored by measuring the optical density at 650 nm (13) at the different temperatures, and in the presence of sodium chloride, artificial gastric juice(pH 2.5), and artificial 0.3% bile salts (14). Morphology was examined by SEM and physiology by the characteristics as given in Bergey's Manual of Systematic Bacteriology (15).

In addition to the physiological traits, carbohydrate fermentation of the isolates was identified by using API 50 CH strips and API 50 CHL medium (BioMérieux, Marcy, l'Etoile, France) at 30°C for 48 hr according to the manufacturer's instructions. The results obtained were read by using the APIWEB version 5.0 (BioMérieux). Lactic acid configuration was determined by an enzymatic method by using a D/L-Lactic acid Enzymatic Bioanalysis kit (Boehringer-Mannheim, Mannheim, Germany). Scanning electron microscopic (SEM) pictures of the isolates were taken with a field emission scanning electron microscope (Model 50A-MRH, Japan) according to the method described earlier (16).

rRNA-based molecular typing experiments For PCR experiment, total DNA was prepared by using the Qiagen DNA purification kit (Qiagen, Valencia, Germany) according to the manufacturer's instructions. Cells from 1 mL of overnight grown MRS culture were precipitated by centrifugation, washed and resuspended in sterile deionized water. PCR amplification was performed in a 25 μL reaction volume containing the following reagents (17): 1 × *Taq* buffer, 0.5 units of *Taq* DNA polymerase (New England Biolab, Beverly, MA, USA), 25 ng of each primer, 0.5 μL of bacterial suspension and 0.1 mM of dNTP (Amersham Biosciences, Uppsala, Sweden). The species-specific primers for *L. brevis* were used (18): The sequence of the forward primer was 5'-CTTGCACTGAT TTTAACA-3', and for the reverse primer was 5'-GGGCG GTGTGTACAAGGC-3'. Polymerase chain reactions were carried out in a programmable thermal cycler (PTC-100, MJ Research, USA), with the following steps: one cycle of denaturation at 94°C for 2 min, 25 cycles of 94°C for 1 min, 40°C for 1 min and 72°C for 1 min were performed. Final extension was carried out at 72°C for 10 min. Amplified PCR products were run on 1%(w/v) agarose gel in 0.5x Tris-borate-EDTA buffer solution at 100V for 40 min. When needed, the PCR products were digested with *Pst*I (Promega, Madison, WI) and run on agarose gel as described above. Both the amplified products and restriction digests were run on 1.5%(w/v) agarose gel and stained with 0.5 g/mL ethidium bromide (Sigma). A 100 bp ladder (Promega) was used as a size marker.

Antibiotic sensitivity test Antibacterial susceptibility test was carried out by using BBL Sensi-Disc Susceptibility Test Disc (Becton, Dickinson and Company, Sparks, MD, USA) according to the manufacturer's recommendations.

Isolation of plasmid DNA Cells were grown upto the late logarithmic stage in MRS broth containing 0.5% glucose, and the plasmid DNA was extracted by Atmanbio plasmid mini-prep kit (Atmanbio, Takarakorea Biomedical Inc, Korea) according to the manufacturer's instructions. After treated with RNase (Sigma) to remove RNA, the DNA was subjected to digestion with some restriction endonucleases (Takarakorea) as per the instructions given in supplier's guide and run on 1% agarose according to the similar procedure as described above. The size of plasmid DNA on agarose gel electrophoresis was calculated by the reference size of the 8 plasmid bands of *E. coli* V517 (19).

Results

Isolation and identification of the LAB isolates from *kimchi* Out of about 3,000 colonies formed on the MMRS agar surface after 98 home-made Chinese cabbage *kimchi* samples were spread, eight distinct LAB isolates were finally selected based on their inhibitory activities against *L. monocytogenes*, a fatal pathogen causing foodborne listeriosis (9) (Table 1). As a result, a strain, YK005 was found to possess highest inhibitory activity

Table 1. Antibacterial activities of the LAB isolates from *kimchi*

Kimchi isolates	Antibacterial activity	
	L. monocytogenes KCCM 40307	*E. coli* O157:H7 ATCC 35150
YK001	+[a]	-
YK005	+	+
YK008	+	-
YK011	+	-
YK012	+	-
YK013	+	-
YK014	+	-
YK015	+	-

[a](+) indicates the clear zone longer than 14mm and (-) indicates the zones less than 14mm in diameter.

against the gram-positive *L. monocytogenes* (Fig. 1a) and weak activity against gram-negative *E. coli* O157:H7 (Table 1, Fig. 1b). It was observed that this trait was very uncommon for most of the gram-positive bacteriocin producing LAB, since bacteriocins are defined as a substance which are inhibitory to the organisms closely related to the producer bacteria (12). The isolate YK005 was found to be rod shaped with dimensions of 3.8 μm long × 0.5 μm wide, gram-positive, catalase-negative, non-sporeforming, and gas producing (heterolactic) bacteria (Fig. 2). For identification, biochemical data obtained by using the API 50 CHL medium presumably suggested that this strain was a strain of *L. brevis* and upon matching with a reference strain, *L. brevis* KCCM 35464, exhibited 99% matching level. As shown in Fig. 3, the data from an rRNA-based typing experiment with a pair of species-specific primer of *L. brevis* also led to the positive identification of *L. brevis*, since a single band of 1400 bp was commonly observed in the amplicons (18). The fingerprints, by digestion of the PCR products with *Pst*I into two separate bands of 550 and 850 bp, were in good line with those obtained with *L. brevis* KCCM 35464 as a reference strain. Accordingly, the isolate YK005 was tentatively named as *L. brevis* YK005.

Effect of NaCl concentrations The time for the optimum ripening of *kimchi* varies depending upon the fermentation temperature and salt concentration. The level of NaCl in the MMRS broth affected the growth of the 8 LAB isolates at 30°C. As shown in Table 2, all the isolates were salt-resistant up to 8%(w/v) level but none of the iso-

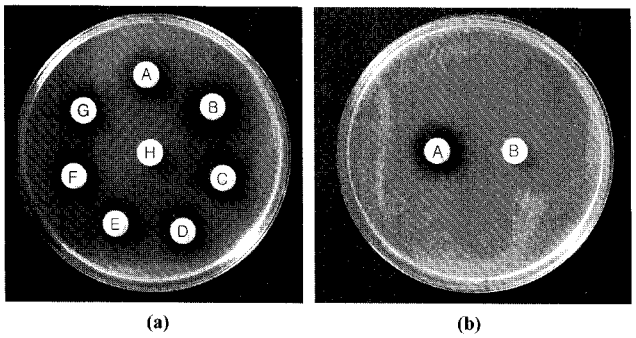

Fig. 1. Antilisterial activities of the LAB isolates from *kimchi*. (a) A : YK001, B : YK005, C : YK008, D : YK011, E : YK012, F : YK014, G : YK015 , H : Control, Test organism: *L. monocytogenes* KCCM 40307. (b) Antilisterial activity of YK005 against *E. coli* O157:H7. A: 10X Concentrated culture supernatant (pH 6.5); B: Culture broth (Control).

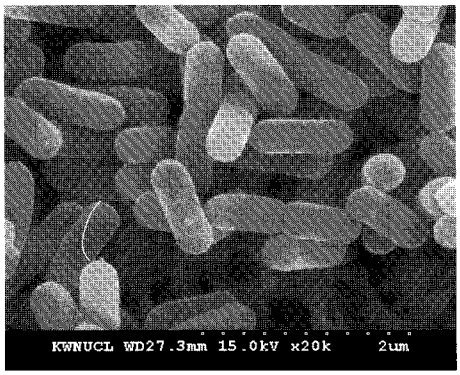

Fig. 2. Scanning electron micrograph of YK005 (length: 3.8 μm, width: 0.5 μm).

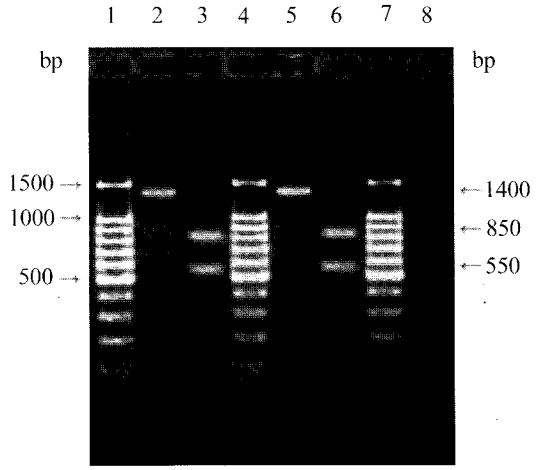

Fig. 3. PCR products of total bacterial DNA with the *L. brevis*-specific primer. Strains of *L. brevis* are KCCM 35464 (lanes 2, 3) and YK005 (lanes 5, 6). Lanes 2, 5: the 1400 bp products after PCR amplification with the *L. brevis*-specific primer (18); lanes 3, 6: the 1400 bp fragment cut with *Pst*I. Lane 8: negative control (no DNA). Lanes 1, 4, and 7: molecular size DNA marker 100 bp plus DNA ladder (Promega). Arrows highlight the uncut 1400 bp product and its two *Pst*I fragment sized 850 and 550 bp.

Table 2. Growth of antilisterial LAB isolates at different NaCl concentrations in the MMRS broth at 30°C

kimchi isolates	NaCl (%)				
	6	7	8	9	10
YK001	+	+	+	-	-
YK005	+	+	(+)	-	-
YK008	+	+	+	(+)	-
YK011	+	+	(+)	-	-
YK012	+	+	+	(+)	-
YK013	+	+	(+)	-	-
YK014	+	+	(+)	-	-
YK015	+	+	(+)	-	-

Growth was monitored daily at the different temperatures for two weeks: * +, actively grown; (+), slightly grown; -, not grown.

lates were grown at 10%$_{(w/v)}$ NaCl. As expected, the growth of YK005 was very slow in the presence of 8% NaCl.

Viability in the artificial gastric and bile juice In general, adult stomach is very acidic as it has a low pH 1.4-2.0 which is sufficient enough to inactivate most of bacteria, but *in vivo* the bacterial death is lowered due to buffering capacity of foods (20). Viability tests were conducted in the presence of gastric juice, pH 2.5 and 0.3% artificial bile salts for the isolates in order to evaluate a potential probiotic function. After exposure to artificial gastric juice of pH 2.5 for 9 hr, the optical density reached above 0.1 O.D$_{650}$ with the variable tolerance for all of the tested strains as shown in Table 3. In other words, all the strains had weak resistance to artificial gastric juice and no more growth was observed after 27 hr incubation. On the other hand, bacterial growth was maintained at high level for 18 hr immediately after 9 hr pre-incubation in the presence of the artificial bile salts of oxgall (0.3%), as shown in Table 4.

Antibiotic sensitivity test The antibiotic sensitivity of *L. brevis* YK005 is shown in Fig. 4. This particular strain was found to be resistant against colistin and streptomycin, and sensitive to erythromycin, ampicillin and oxytetracycline; while *Leu. mesenteroides*, *L. plantarum*, *L. casei*, and *L. salivarius* bear an intrinsic resistance towards vancomycin (21). In this context, potential application of *L. brevis* YK005 as human probiotic could be suggested.

Table 3. Bacterial growth of the antilisterial LAB isolates in the MMRS broth containing the artificial gastric juice, pH 2.5

Kimchi isolates	Optical density at 650 nm[1)]				pH of gastric juice
	9 hr	18 hr	27 hr	36 hr	
YK001	0.104	0.132	0.143	0.143	2.5
YK005	0.103	0.139	0.155	0.155	2.5
YK008	0.091	0.133	0.156	0.156	2.5
YK011	0.125	0.160	0.180	0.180	2.5
YK012	0.109	0.143	0.164	0.164	2.5
YK013	0.113	0.150	0.187	0.187	2.5
YK014	0.110	0.151	0.164	0.164	2.5
YK015	0.166	0.110	0.148	0.148	2.5
Control[2)]	1.352	7.654	8.450	8.450	2.5

[1)]Optical density at 650 nm was determined in triplicate and the mean value was calculated. Medium pH was adjusted to 2.5 with 0.1N HCl.
[2)]YK005 was incubated in the MMRS broth without artificial gastric juice, pH 2.5.

Table 4. Bacterial growth of the antilisterial LAB isolates in MMRS broth containing the artificial bile salts[1)]

Kimchi isolates	Optical density at 650 nm[2)]			
	9 hr	12 hr	15 hr	18 hr
YK001	1.366	1.410	1.421	1.457
YK005	1.441	1.462	1.487	1.502
YK008	1.327	1.345	1.446	1.471
YK011	1.410	1.417	1.665	1.847
YK012	1.389	1.435	1.694	1.859
YK013	0.173	0.184	0.403	0.540
YK014	0.046	0.062	0.331	0.652
YK015	1.364	1.390	1.668	1.941
Control[3)]	1.352	7.654	8.154	8.450

[1)]Artificial bile salts were prepared in MMRS broth containing 0.3% (w/v) oxgall.
[2)]Optical density at 650 nm was determined in triplicate and the mean value was calculated.
[3)]YK005 was incubated in the MMRS broth without the artificial bile salts.

Discussion

Reportedly, there are more than 200-kinds of *kimchi* products manufactured in Korea. According to a national nutrition survey in 2000, an adult Korean consumes 50-100 g of *kimchi* daily in summer and 150-200 g throughout the winter season (1). *Kimchi* is originally prepared by natural fermentation, and hence is difficult for the industry to keep the product quality consistent. Thus, for establishment of a starter-added technology in the fermented vegetable industry, many researchers have currently focused on determining predominant microorganisms involved in the fermentation and examining their effects on the fermentation process (22). To explore the major types of LAB involved in the initial and/or the middle stage of manufacturing of *kimchi*, 8 LAB isolates, which were most commonly recovered from the *kimchi* samples of pH above than 4.0 were selected for identification by using both a conventional biochemical identification method based on carbohydrate utilization and few molecular typing methods including ITS-PCR (23-24), RAPD-PCR (25) and 16s rRNA gene sequence analysis (26). The results revealed that biochemical test (API 50 CH strip) coincided with that of 16s rDNA sequencing data by about 60% at genus-level and about 40% at species-level (data not shown). More recently, *Leu. citreum* IH22, one of the dominant strains found during the early and mid-phases of *kimchi* fermentation, was tentatively used as a starter for *kimchi* fermentation (27). The strain was consistently over than 95% of the population in the IH22-treated *kimchi* over the 5-day fermentation period, while heterogeneous LAB were observed in the control group. In this respect, antilisterial

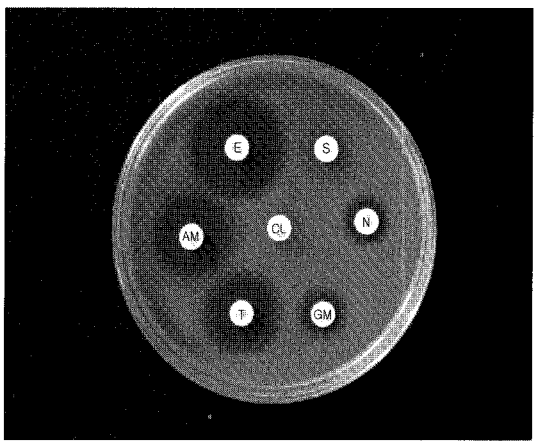

Fig. 4. Determination of antibiotic sensitivity of *L. brevis* YK005. Erythromycin (E) 15μg, Streptomycin (S) 10μg, Neomycin (N) 10 μg, Gentamycin (GM) 10 μg, Oxytetracycline (T) 30 μg, Ampicillin (AM) 10 μg, Colistin(CL) 10 μg.

L. brevis YK005 should be tested for its survival during *kimch* fermentation prior to application as a starter for production of the commercial products.

LAB produces several antagonistic systems such as hydrogen peroxide, diacetyl, organic acids, and bacteriocins (28). There have been some bacteriocins active against *Listeria*, like nisin by *Lactococcus. lactis*, mesenterocins or leucosins by *Leu. mesenteroides*, and pediocions by *P. acidilactici* (29). For bacteriocin production, importance of pH adjustment of MRS broth in bacterial growth and bacteriocin production has been mentioned in the previous studies (30).

Mheen and Kwon (3) showed that temperature played a critical role in the formation of bacterial flora in during the fermentation of *kimchi*, and the fermentation was faster at high temperature and low salt content than at low temperature and high salt content. Lim *et al.* (11) claimed that *Lactobacillus* spp. (59.7%) was a major flora for *kimchi* fermentation at 25°C and *Leuconostoc* spp. were the major one at 5°C. In this study, four LAB isolates, YK001, YK011, YK013, and YK014 were able to grow at 5°C but failed to grow at 40°C, suggesting that they belong to the group of psychrotrophic bacteria. Similarly, *L. brevis* YK005 failed to grow at 45°C (data not shown). In contrast to sauerkraut, where salt level is lower than that of *kimchi*, in the present study, *L. brevis* was more frequently detected in *kimchi* at higher temperature and low salt content during the period of over-ripening.

In vitro tolerance to low pH, bile acids, and pancreatic fluids has often been considered as good indicator for survival of the potential probiotic strains through the GI tract after consumption. It is known that bile salts are resistant to proteases and can be degraded only by bile salt hydrolase, which is produced by some enteric bacteria resistant to bile salts. Kim *et al.* (31) investigated bile-salt hydrolyzing activities of the bacterial strains isolated from the fermented milk products. Twenty one strains of the *Lactobacillus* tested were found to be active in terms of mediating their enzymatic functions like degradation of taurocholic and glycocholic acid to cholic acid, suggesting that a yogurt fermented with selected LAB may have an anti-cholesterolemic effect. Moreover, the normal growth of LAB was obtained even though they were slightly suppressed by 0.3% bile salt and the cellular permeability in the presence of bile salt increased significantly. As far as acid tolerance was considered, most of the isolates survived with the variable tolerance depending on the tested strains after exposure to artificial gastric juice (pH 2.5) for 9 hr. Lee and No (20) reported that 144 out of 157 *kimch* isolates survived for 24 hr in the artificial bile after 3 hr incubation in the artificial gastric juice (pH 2.5) for 3 hr. Kang *et al.* (14) reported that *L. acidophilus* a-4 survived at pH 2.5 for 3 hr without any detectable loss of viability, and above 75% viability was observed for *L. acidophilus* a-2 and *L. casei*. The results (Table 3-4) obtained in our present study are in fairly good accordance with the ones reported previously.

More recently, the S-layer has been shown to mediate the adherence of *L. brevis* strain to human intestinal, urinary bladder, and endothelial cells (32). Accordingly, better adhesion properties could assist the competitive exclusion of potentially harmful microbes by *L. brevis*. In this context, application of this strain for probiotic purposes could be suggested. As reported earlier, the genus *Lactobacillus* harbors many plasmids of different molecular size (33). Presence of plasmid DNA is mostly reported to be associated with bacterial antibiotic resistance. However, information on the drug resistance of the commercial *Lactobacillus* strains is very limited. Vancomycin resistance is also reported to be spread by a transposon, Tn 1546, carrying the *van* (A) gene cluster (34). The macrolide resistance genes most frequently found in the bacterial isolates from animals and human are the *erm* genes (35). Studies have shown that *L. delbrueckii* subsp. *bulgaricus* strains, an yogurt starter culture, has an intrinsic resistance towards mycostatin, nalidixic acid, neomycin, polymixin B (colistin), trimethoprim, colymycin, sulfamethoxazol, and sulfonamids (36). Only a few papers have described the successful transfer of plasmid DNA between *Lactobacillus* strains (37). Recently, the acquisition and incorporation of the *tet*(M) gene into the plasmid, pMD5057, of a *Lactobacillus* species was successfully exercised (38). As shown in Fig. 5, *L. brevis* YK005 was found to possess a single plasmid DNA of size 35.8 kb, which was bigger than the plasmids of size 10 to 12 kb of *L. brevis* GRL1 (39). In this regards, *L. brevis* YK005 was screened for the occurrence of Bac^- variants in the presence of acriflavine (20 μg/mL), as a curing agent, following repetitive transfers at 37°C. However, we failed to detect Bac^- variants from the culture. Therefore, it is uncertain whether or not the antilisterial function was associated with the plasmid DNA of YK005. In the previous paper, plasmid profile analysis of *L. sake* Lb706 indicated that a plasmid of about 18 MDa may be involved in the formation of bacteriocin and in imparting immunity to this antibacterial compound (30). van Reenen *et al.* (40) reported that high DNA homology was obtained between the plasmid DNA of *L. plantarum* 423 and the pediocin PA-1 operon of *P. acidilactici* PAC1.0, suggesting that plantaricin 423 is plasmid-encoded and is related to the pediocin gene cluster. Since, DNA-relatedness between YK005 and a reference *L. brevis* strain was not examined

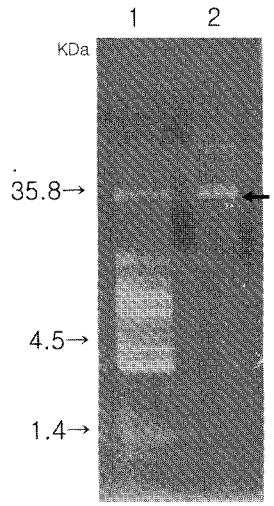

Fig. 5. Plasmid DNA profiles of *L. brevis* YK005. Lanes: 1, molecular size marker of *E. coli* V517; 2, plasmid DNA of *L. brevis* YK005.

in this study, further study is needed to state whether or not the antilisterial function is associated with the plasmid DNA of YK005. For better understanding of the antilisterial activity of *L. brevis* YK005, additional studies should be performed on identifying the factors responsible for its production, purification, and mode of antibacterial actions.

Acknowledgments

This work was supported by Yonsei University Research Fund of 2002 and in part by a Maeji Academic Research Grant, 2004.

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